

## Quantitative genome-wide methylation analysis of high-grade non-muscle invasive bladder cancer

Kitchen, Mark; Bryan, Richard; Emes, Richard D; Glossop, John; Luscombe, Christopher; Cheng, Kar; Zeegers, Maurice; James, Nicholas; Devall, Adam; Mein, Charles A; Gommersall, Lyndon; Fryer, Anthony; Farrell, William E

DOI:

[10.1080/15592294.2016.1154246](https://doi.org/10.1080/15592294.2016.1154246)

License:

Other (please specify with Rights Statement)

*Document Version*

Peer reviewed version

*Citation for published version (Harvard):*

Kitchen, M, Bryan, R, Emes, RD, Glossop, J, Luscombe, C, Cheng, K, Zeegers, M, James, N, Devall, A, Mein, CA, Gommersall, L, Fryer, A & Farrell, WE 2016, 'Quantitative genome-wide methylation analysis of high-grade non-muscle invasive bladder cancer', *Epigenetics : official journal of the DNA Methylation Society*, vol. 11, no. 3, pp. 237-246. <https://doi.org/10.1080/15592294.2016.1154246>

[Link to publication on Research at Birmingham portal](#)

### **Publisher Rights Statement:**

This is an Accepted Manuscript of an article published by Taylor & Francis in *Epigenetics* on 01/03/2016, available online: <http://www.tandfonline.com/10.1080/15592294.2016.1154246>

### **General rights**

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

### **Take down policy**

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact [UBIRA@lists.bham.ac.uk](mailto:UBIRA@lists.bham.ac.uk) providing details and we will remove access to the work immediately and investigate.

**Quantitative genome-wide methylation analysis of high-grade non-muscle  
invasive bladder cancer.**

Mark O Kitchen<sup>\*1,2</sup>, Richard T Bryan<sup>3</sup>, Richard D Emes<sup>4</sup>, John R Glossop<sup>1</sup>, Christopher  
Luscombe<sup>2</sup>, KK Cheng<sup>3</sup>, Maurice P Zeegers<sup>3,5,6,7</sup>, Nicholas D James<sup>8</sup>, Adam J Devall<sup>3</sup>,  
Charles A Mein<sup>9</sup>, Lyndon Gommersall<sup>2</sup>, Anthony A Fryer<sup>1</sup>, William E Farrell<sup>1</sup>.

(1) Institute for Science and Technology in Medicine, Keele University, UK.

(2) Urology Department, University Hospitals of North Midlands NHS Trust, UK.

(3) Institute of Cancer and Genomic Sciences, University of Birmingham, UK.

(4) Advanced Data Analysis Centre, University of Nottingham, UK.

(5) Department of Complex Genetics, Maastricht University Medical Centre, The  
Netherlands.

(6) NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht  
University Medical Centre, The Netherlands.

(7) CAPHRI School for Public Health and Primary Care, Maastricht University Medical  
Centre, The Netherlands.

(8) Cancer Research Unit, University of Warwick, UK.

(9) The Genome Centre, Barts and the London School of Medicine and Dentistry, London,  
UK.

\* Corresponding author.

## Abstract

High-grade non-muscle invasive bladder cancer (HG-NMIBC) is a clinically unpredictable disease with greater risks of recurrence and progression relative to their low-intermediate-grade counterparts. The molecular events, including those affecting the epigenome, that characterise this disease entity in the context of tumour development, recurrence and progression, are incompletely understood. We therefore interrogated genome-wide DNA methylation using HumanMethylation450 BeadChip-arrays in 21 primary HG-NMIBC tumours relative to normal bladder controls. Using strict inclusion-exclusion criteria we identified 1,057 hypermethylated CpGs within gene promoter-associated CpG islands, representing 256 genes. Bisulphite Pyrosequencing validated the array data and examined 25 array-identified candidate genes in an independent cohort of 30 HG-NMIBC and 18 low-intermediate-grade NMIBC. These analyses revealed significantly higher methylation *frequencies* in high-grade tumours relative to low-intermediate-grade tumours for the *ATP5G2*, *IRX1* and *VAX2* genes ( $p<0.05$ ), and similarly significant increases in *mean levels* of methylation in high-grade tumours for the *ATP5G2*, *VAX2*, *INSRR*, *PRDM14*, *VSX1*, *TFAP2b*, *PRRX1*, and *HIST1H4F* genes ( $p<0.05$ ). Although inappropriate promoter methylation was not invariantly associated with reduced transcript expression, a significant association was apparent for the *ARHGEF4*, *PON3*, *STAT5a*, and *VAX2* gene transcripts ( $p<0.05$ ). Herein, we present the first genome-wide DNA methylation analysis in a unique HG-NMIBC cohort, showing extensive and discrete methylation changes relative to normal bladder and low-intermediate-grade tumours. The genes we identified hold significant potential as targets for novel therapeutic intervention either alone, or in combination, with more conventional therapeutic options in the treatment of this clinically unpredictable disease.

**Key words:** *High-grade Non-Muscle Invasive Bladder Cancer, Epigenetics, Methylation, HumanMethylation450 BeadChip Array, Gene Expression*

## Introduction

Bladder cancer is the ninth most common cancer worldwide.<sup>1</sup> The majority of bladder cancers are transitional cell carcinomas (TCC), of which 70-80% are non-muscle invasive (NMIBC) at presentation.<sup>2</sup> Poorly differentiated 'high-grade' (HG)-NMIBC is a clinically important sub-type, accounting for approximately 10-15% of all NMIBCs at presentation.<sup>3, 4</sup>, These high-grade tumours are typically more aggressive than their low- and intermediate-grade counterparts, manifest by higher rates of recurrence and progression to invasive and metastatic disease despite intensive and prolonged intravesical treatment.<sup>5, 6</sup>

The majority of NMIBCs are thought to be consequent to, and represent initiation and progression from, a complex interplay between sporadic, environmental, and heritable risk factors, including those that impact upon genetic and epigenetic pathways. NMIBCs and muscle invasive bladder cancers (MIBCs) have been shown to develop independently ('the two pathway model') on the basis of gain of function fibroblast growth factor receptor 3 (*FGFR3*) mutations in NMIBC, and loss of function mutations in retinoblastoma 1 (*RB1*) and tumour protein 53 (*p53*) in MIBC,<sup>7-10</sup> and have been shown to evolve from different cell types.<sup>11, 12</sup> However, the molecular pathways responsible for the evolution, outgrowth and progression of HG-NMIBC have not been subject to comprehensive study or investigation; indeed, it is currently unclear whether HG-NMIBCs arise as a discrete disease entity, whether they represent step-wise progression from low-intermediate-grade NMIBC tumours, or whether they sit at a molecular crossroads between NMIBC and MIBC.<sup>7, 13 11</sup> This uncertainty is illustrated by the findings that high-grade tumours harbour abnormalities in common with low-intermediate-grade NMIBC, such as mutations of *FGFR3* and/or rat sarcoma viral oncogene homolog (*RAS*) pathway genes<sup>14, 15</sup>, but also display extensive genetic instability and compromised regulation of vital cellular processes more in keeping with MIBC.<sup>14, 16</sup>

Epigenetic modifications are frequently implicated in the development of human malignancies, and in these cases, are typically apparent as inappropriate gene promoter CpG island DNA methylation, histone tail modification(s), aberrant expression of micro- and long non-coding-RNAs, and less frequently, loss of gene body/intergenic methylation.<sup>17, 18</sup> These heritable modifications, or *epimutations*, impact upon gene expression either alone or in combination, and promote tumour evolution and/or progression by suppressing the expression of growth inhibiting and/or apoptosis promoting genes, and less frequently by leading to relaxed control of expression of growth promoting genes.<sup>17, 19, 20</sup>

Epigenetic modifications and associated gene silencing have been shown in NMIBC, and specific patterns of DNA methylation, histone modifications and microRNA expression have been reported as associated with tumour growth characteristics, patient/clinical outcomes and with field defect phenomena.<sup>21, 22</sup> However, the majority of these reports have described epigenetic changes in heterogeneous populations of NMIBC, with an abundance of low- and intermediate-grade tumours relative to high-grade tumours. With the exception of our recent candidate-gene study<sup>23</sup> and a single report investigating the Myopodin A gene<sup>24</sup>, HG-NMIBCs have not been considered as a discrete entity for the investigation of epigenetic modifications.

In this study, we interrogated DNA methylation on a genome-wide scale using methylation BeadChip-array technology, in a unique cohort of HG-NMIBCs. Through comparisons with methylation levels and gene-expression in low/intermediate-grade tumours, we extend the current understanding of bladder cancer tumourigenesis and identify potential epigenetic mechanisms implicated in the development of high-grade NMIBC, and those that might represent novel therapeutic drug-targets.

## Results

### *Technical Validation of array by Pyrosequencing:*

Subsequent to array processing, normalisation and peak-based correction (see patients and methods), a technical validation was performed by comparing array-derived  $\beta$ -values with Pyrosequencing-derived methylation values. Across 120 data-points (5 CpGs, 24 samples) encompassing a broad range of array  $\beta$ -values, a strong positive correlation was found between the methylation values (Spearman's rank correlation  $r=0.912$ ,  $p<0.00001$ ; **Supplemental Figure S1**).

### *In-house filtering criteria:*

CpGs showing differential methylation in HG-NMIBC relative to normal bladder controls were identified following a series of stringent filtering criteria, as described previously and shown in **Figure 1**.<sup>25, 26</sup> On the basis of these criteria, a total of 1,057 CpGs, representing 256 genes, were identified as hypermethylated ( $\geq 0.4$   $\beta$ -value increase) in 15 or more of the 21 high-grade tumours, relative to their mean values in the normal bladder controls.

### *Hierarchical clustering analyses:*

The filtered dataset was next subject to unsupervised hierarchical cluster analysis (**Figure 2**): the high-grade tumours cluster independently from the normal bladder control samples. In these cases, methylation is barely detectable within the normal bladder samples, whereas 15 or more of the high-grade tumours show inappropriate methylation across all 1,057 CpG dinucleotides, spanning 256 gene-promoter-associated CpG islands (**Supplemental Table S2**).

*Independent validation by Pyrosequencing:*

We next selected 25 genes for independent validation by Pyrosequencing on the basis of their frequent methylation in the discovery cohort that comprised 21 high-grade tumours. These analyses revealed similar frequencies and mean levels of methylation as those apparent from the BeadChip array for 24 of the 25 genes. As further confirmation, we extended the Pyrosequence analyses to an independent investigation cohort of 30 HG-NMIBC tumours. Similar frequencies and mean levels of methylation between the discovery and investigation cohorts reinforced our confidence in the array-derived data (**Supplemental Table S3**). At this stage, and to assess for potential confounders, we assessed associations between patient demographic data and methylation patterns across these 25 genes, using separate multivariate models. No correlations were identified in these analyses, suggesting demographic factors did not significantly impact upon the methylation patterns identified (data not shown).

*Differential subtype-specific promoter methylation in NMIBC:*

We next determined methylation across the 25 genes described above in HG-NMIBC relative to that apparent in low-intermediate-grade tumours and in comparison to normal bladder controls (**Supplemental Table S4**). Similar to other groups<sup>27 28</sup>, we displayed these methylation data, across the high-grade and low-intermediate-grade tumours and normal controls, by heatmap (**Figure 3**). This demonstrated heterogeneous patterns of methylation across the 51 high- and 18 low-intermediate-grade tumours relative to the normal bladder controls. Gene-specific differences in methylation were apparent between the high-grade tumours and their low-intermediate-grade counterparts on visual inspection. Closer examination of these data showed that the differences appeared to impact on either the relative frequency and/or the mean levels of methylation between these tumour subtypes. As

examples of these differences, the ten most differentially methylated genes are shown in **Table 1**.

*Methylation frequencies in high- and low-intermediate-grade tumours:*

For ten of the genes we took forward for further analyses (*ATP5G2*, *HIST1H4F*, *INSRR*, *IRF8*, *IRX1*, *PRDM14*, *PRRX1*, *TFAP2b*, *VAX2* and *VSX1*), there was an higher frequency of methylation in high-grade tumours versus low-intermediate grade tumours (**Table 1**). Moreover, the increases were statistically significant for the *ATP5G2*, *VAX2* and *IRX1* genes ( $p < 0.05$ ), and approached significance for the *INSRR*, *IRF8*, *PRDM14* and *VSX1* genes.

*Mean levels of methylation in high- and low-intermediate-grade tumours:*

The mean levels of methylation in the high-grade tumours were next assessed by Pyrosequencing (right-sided panel of **Table 1**, and **Figure 4**); for eight of the ten genes, mean levels of methylation were significantly greater in high-grade tumours relative to their low-intermediate-grade counterparts. In addition, and as low-intermediate-grade tumours were not subject to array analyses relative to normal bladder, further pairwise-testing was performed. This analysis identified significant differences between mean levels of methylation in the low-intermediate-grade tumours and normal bladder in four of the ten genes assessed. The range, distribution and mean levels of methylation are shown in **Figure 4**, and show for each of the genes, a stepwise trend toward increasing methylation from normal bladder to low-intermediate and high-grade tumours.

*Methylation-Associated Changes in Gene Expression:*



Across the high-grade NMIBC tumours, sufficient sample was available for gene expression analyses for 17 of the 25 genes. With the exception of the *ARHGEF4* gene, promoter-associated CpG island methylation was negatively correlated with transcript expression for all genes assessed (data not shown). Furthermore, the presence of promoter methylation was significantly correlated with reduced transcript expression for the *PON3*, *STAT5a* and *VAX2* genes (Spearman's correlation coefficients -0.60, -0.50 and -0.48 respectively, all  $p < 0.05$ ). Conversely, promoter methylation was significantly positively correlated with gene transcript expression for the *ARHGEF4* gene (Spearman's correlation coefficient 0.62,  $p < 0.05$ ). **Figure 5** shows the expression levels for these four genes across the high-grade tumours.

#### *Gene Ontology analysis of inappropriately methylated genes:*

Gene Ontology analyses of the 256 differentially methylated genes identified 'over-representation' of multiple categories of biological processes, molecular functions and pathways. In particular, highly significant over-representation was identified for specific biological processes, including regulation of RNA polymerase II activity and DNA transcription, and for pathways involving cell adhesion and PI3K-Akt signalling (**Supplemental Table S5**).

## Discussion

In common with most other tumour types, bladder cancers harbour epigenetic aberrations which are frequently apparent as inappropriate DNA methylation.<sup>8, 22, 29</sup> However, reports are limited and largely confined to heterogeneous patient cohorts of NMIBC or MIBC;<sup>30</sup> despite their clinical importance, high-grade NMIBC tumours are rarely investigated as a discrete entity in the context of disease and/or subtype-specific epigenetic modifications.<sup>23</sup> To address this, we performed genome-wide analyses of DNA methylation using BeadChip array technology in high-grade NMIBC, comprising a discrete cohort of tumours recruited at initial presentation. This analysis, the first '450K array' interrogation in bladder cancer, revealed multiple and novel frequently differentially methylated genes in these tumours relative to normal bladder. Through Pyrosequence analysis of sodium bisulphite converted DNA, we extended our analyses to include independent cohorts of high- and low-intermediate-grade tumours. These investigations confirmed the array-derived data for the high-grade tumours, and showed them as harbouring significantly increased frequencies and/or mean levels of gene-specific methylation relative to low-intermediate-grade tumours. Moreover, for some of the genes investigated, a significant inverse correlation between promoter methylation and gene expression levels was apparent and suggests their potential as targets for therapeutic intervention.<sup>29 31 32</sup>

Initially we performed a technical validation of the discovery cohort data by Pyrosequence analysis of converted DNA.<sup>25 33 34</sup> In common with previous reports and across multiple genes, these analyses confirmed and reinforced the array-derived data.<sup>34 35 36</sup> These analyses also showed that for the majority of regions investigated, methylation extended to include contiguous promoter-associated CpG sites. On the basis of previous reports from our own and other groups,<sup>37 38</sup> we employed stringent criteria ( $\beta$ -value differences  $\geq 0.4$ ) to identify differentially methylated genes across multiple CpG sites; such criteria are more

consistently associated with *bona fide* changes in methylation, and are more likely to show associations with gene expression.<sup>37, 39 40, 41</sup>

The analysis of the discovery cohort of high-grade NMIBC identified 1,057 CpGs, across 256 gene-promoter-associated CpG islands. Cluster analysis and heat map display of these regions revealed extensive and frequent differential methylation in the tumours relative to normal bladder controls. As our study represents the first 450K analysis of high-grade bladder cancer a direct 'like-for-like' comparisons of our findings with those of other groups was not possible; however, the number of differentially methylated sites we identified appeared to be lower than those previously reported in other tumour types.<sup>42 43</sup> Potential explanations for these findings are the tumour type *per se* and/or the stringency of our inclusion-exclusion criteria and definition of differential methylation.<sup>44</sup>

For the genes identified, we performed gene ontology and KEGG pathway analyses. In these cases we identified significant over-representation of genes in processes and pathways previously reported by other groups as subject to epigenetically-mediated dysregulation in tumour development. For examples, these included transcription and cell signalling and adhesion<sup>45-47</sup>, suggesting possible similar roles in high-grade bladder tumours, and their validity as targets for further investigation.

We next extended our investigation of multiple novel genes to an independent cohort of high-grade tumours, and a cohort of low-intermediate-grade tumours for comparison. Similar frequencies and mean levels of methylation, as determined by Pyrosequence analysis, were apparent within the discovery and investigation cohorts of high-grade tumours, suggesting our approach for the identification of candidates by array analysis was robust. Interestingly, many of the genes identified as novel and differentially methylated were also inappropriately methylated in low-intermediate-grade tumours. However, and despite the absence of genes as being exclusively associated with either high- or low-intermediate-grade tumours, the frequency and mean levels of gene-promoter methylation in the high-grade tumours were

significantly higher than in the low-intermediate-grade tumours. Indeed, similar observations with respect to differences in the frequencies of methylation between high- and low-grade bladder tumours were first suggested by Ibragimova *et al.*<sup>47</sup> Similar subtype and/or grade-associated differences have been reported in other tumour types including, pituitary, breast, and colon cancer subtypes.<sup>37, 48, 49</sup> In our analysis of NMIBC it remains unclear whether the increase in frequency and/or mean levels of methylation in the more aggressive tumours represents a more rapid accumulation of epigenetic changes during tumour progression, or reflects distinct epigenetic pathways of tumour development and outgrowth.<sup>50, 51</sup> Our findings may therefore reflect either of the described scenarios in the more aggressive (high-grade) tumours and suggests that these tumours are either consequent to progression from low-intermediate-grade tumours, or are the progeny of aberrations in distinct epigenetic pathways within these NMIBC subtypes. Moreover, the identification of different patterns of methylation between tumours represents an important area for future investigation. In this case, methylation may hold promise as an 'at diagnosis' biomarker of long-term tumour outcome, similar to that described in colorectal, breast and lung cancers.<sup>52-54</sup>

Although many of the novel genes we identified have not been previously reported in bladder cancer, their inappropriate methylation, accompanied with gene-silencing, has been reported in the context of other tumour types and suggests potential roles as tumour suppressor genes.<sup>55, 56 57</sup> To determine associations between methylation and gene expression, we confined our studies to genes showing frequent and/or high mean levels of methylation. For the majority of gene-transcripts we investigated, promoter methylation was negatively correlated with reduced transcript expression, although not significantly so (data not shown). However, as described by our own and other groups, this may reflect a passenger-driver phenomenon where, in the 'passenger' context, gene expression is not directly influenced by the observed epigenetic modification(s).<sup>58 59</sup> However, for four of seventeen transcripts we examined, significant correlations between methylation and transcript expression were apparent. In these cases, and for the *PON3*, *STAT5a* and *VAX2*

genes, promoter methylation was significantly associated with reduced gene expression, whilst the converse was true for the *ARHGEF4* gene. Such associations are similar to those described previously in multiple other cancers and in NMIBC.<sup>20, 21 43</sup> Indeed, for two of these genes, *PON3* and *STAT5a*, previous studies in mice and cell-line models have described potential tumour suppressor roles.<sup>60 61</sup> If this is the case, then these genes may represent important targets for further studies of functional the significance of methylation and reduced expression in a bladder tumour context, including *in-vitro* investigations of de-methylating agents designed to restore gene expression.

In summary, we have presented the first comprehensive genome-wide DNA methylation analysis of NMIBC in a unique cohort of high-grade tumours. The study has reported an increase in the frequency and/or mean levels of methylation at gene promoter-associated CpG islands in high-grade tumours relative to their low-intermediate-grade tumour counterparts, that in some cases is associated with reduced gene expression. These findings suggest that epigenetic modifications, alone or in combination with other aberrations, are causal in the development and/or progression of this tumour type. Further studies are required to assess the functional significance of epigenetic changes in HG-NMIBC; however, we suggest that the genes identified hold significant potential as targets for novel therapeutic interventions alone, or in combination, with conventional therapeutic options in the treatment of this clinically unpredictable disease.

## Patients and methods

### *Human tissue samples*

Primary tumour and normal bladder tissues used were provided by the Bladder Cancer Prognosis Programme (BCPP, National Research Ethics Service East Midlands - Derby 06/MRE04/65.)<sup>62</sup>, the University of Birmingham Human Biomaterials Resource Centre (National Research Ethics Service (North West 5): 09/H1010/75), and the University Hospitals of North Midlands NHS Trust (National Research Ethics Service (South Central – Oxford C): 12/SC/0725). All samples were confirmed histologically as normal bladder urothelium (control,  $n=4$ ), G3pT1 TCC (high-grade: discovery cohort  $n=21$ , investigation cohort  $n=30$ ), and G1/2 pTa/1 TCC (low/intermediate-grade:  $n=18$ ). As previously described<sup>23</sup>, patients received repeat bladder tumour resection (TURBT), cystectomy and/or intra-vesical therapy as recommended by European Association of Urology guidelines.<sup>63</sup> All samples (details are provided in **Supplemental Table S1**) were stored at  $-80^{\circ}\text{C}$  prior to nucleic acid extraction, as described below.

### *DNA extraction and bisulphite modification*

Genomic DNA was extracted from tumour and control tissues using a standard phenol-chloroform procedure<sup>64</sup>, then bisulphite-converted using the EZ DNA Methylation Gold kit (Zymo Research) as we have previously described.<sup>37</sup> Bisulphite-conversion of DNA was confirmed in all cases by successful PCR using primers specific to bisulphite-converted DNA (primer sequences in **Supplemental Table S6**). To increase the relative amount and stability of bisulphite-converted DNA, whole-genome amplification (WGA) was performed as previously described.<sup>37</sup>

## *Illumina 450K Methylation Bead-Array Analyses*

Bisulphite-converted DNA from 21 bladder tumours and three normal controls was hybridised to Infinium-based HumanMethylation450 BeadChip arrays (Illumina, San Diego, CA, USA) to quantify DNA methylation at approximately 480,000 CpG positions across the genome, representing more than 21,000 RefSeq genes. In this case, normal bladder was used as control for consistency with previous array analyses<sup>35, 47, 65</sup>, and also to permit comparisons with earlier reports of non-muscle invasive bladder cancer. Arrays were processed according to the manufacturer's instructions (performed by Barts and the London Genome Centre, UK), as described by us previously.<sup>66</sup>

Raw array data were processed using GenomeStudio software and the bioinformatical platform 'NIMBL', as we<sup>67, 68</sup> and others<sup>69</sup> have described. For each probe, the methylation status was reported as a methylation ' $\beta$ -value', where ' $\beta$ ' is defined as the ratio of the methylated signal intensity over the summed intensity of the methylated and unmethylated signals + 100.<sup>40</sup>  $\beta$ -values range from 0 (unmethylated) to 1 (fully methylated). NIMBL was used to perform 'peak-based' correction, to adjust for potential differences in array probe-type sensitivity previously reported<sup>33</sup>; all comparative analyses of high-grade tumours to normal bladder controls, were performed on peak-based corrected  $\beta$ -values, as described by us previously.<sup>68</sup>

Each array passed quality control assessment based upon the performance of internal controls and the distribution of  $\beta$ -values across all array CpGs. As previously described<sup>68</sup>, and represented by **step 1 of Figure 1**, we excluded all CpGs for which any of the 24 samples displayed: (i) probe detection  $p$ -values  $>0.05$  (unreliable probe data), or (ii) missing  $\beta$ -values (preventing analyses of all samples). We also excluded all CpG loci on allosomes (reducing confounding gender-based methylation differences). We used a series of stringent filtering criteria, shown in **Figure 1** and described in the Results section, to identify

inappropriate methylation, defined as a  $\beta$ -value difference  $\geq 0.4$ , in tumour samples relative to the mean of the normal bladder controls.

Unsupervised hierarchical clustering using average linkage criteria was performed using Genesis software (v1.7.6).<sup>70</sup> Gene Ontology (GO) analyses were performed using <http://geneontology.org/> and <http://gather.genome.duke.edu/>, and Kyoto Encyclopaedia of Genes and Genomes (KEGG) analyses with <http://www.genome.jp/kegg/> online platforms, respectively. Bonferroni correction<sup>71</sup> was employed in all GO and KEGG pathway analyses.

#### *Technical validation of Methylation Bead-Chip Array Data*

Five CpG loci encompassing a broad range of  $\beta$ -values derived from 450k array analyses, were assessed by Pyrosequencing (described below), using identical samples, to independently validate the array data ( $\beta$ -values vs. methylation %). Correlation between the methods was assessed across a total of 120 CpGs using Spearman's rank correlation, as shown in **Supplemental Figure S1**. Primer sequences are provided in **Supplemental Table S6**.

#### *Pyrosequencing<sup>TM</sup> of sodium bisulphite-converted DNA*

Validation of array data (discovery cohort) and further quantitative assessment of methylation in the independent (investigation) tumour cohort were performed by Pyrosequencing of sodium bisulfite-converted DNA, as previously described by us<sup>66</sup>, using a PyroMark Q24 Pyrosequencer, PyroMark Q24 Software 2.0 and PyroMark Gold Q24 Reagents. Dependent on the specific gene, and the density of CpGs within their promoter-associated CpG island, between five and nine consecutive CpG sites were assessed. Promoter methylation was defined in tumours if the mean level of methylation across the assessed CpG island was greater either than four standard deviations (4SD), or 20% above,



the mean of the normal controls.<sup>37</sup> The number of tumours methylated for any given gene describes the *frequency* of methylation, whereas the mean percentage methylation *per se* of all of the CpGs surveyed within a gene describes the *mean level* of methylation.

#### *Quantitative RT-PCR*

Total RNA was extracted from control and tumour samples using a standard guanidinium thiocyanate-phenol-chloroform protocol<sup>72</sup>. Complementary DNA (cDNA) was synthesised as described previously<sup>73</sup>. Thermal cycling using SYBR Green was as previously described<sup>74</sup>, with target genes normalised to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the endogenous control gene (**Supplemental Table S6**). Relative quantification of transcript expression was performed using the  $2^{-\Delta\Delta}$  cycle threshold (CT) method<sup>75</sup>, and as previously described<sup>76</sup>. Reduced transcript expression in a tumour was defined where expression was at least 3-fold lower than the mean level of expression observed in control samples; the converse was true for increased transcript expression.<sup>37 38 77</sup>

#### *Non-Array Informatics and statistics.*

STATA (version 8, Stata Corporation, College Station, TX) was used to analyse methylation and gene expression data in tumour and normal cohorts using Fisher's exact tests (frequency of methylation), Student's t-tests (mean level of methylation), and Spearman correlation coefficients (associations between methylation and gene expression). *p*-values <0.05 were considered statistically significant.

389    **Ethics Committee Approvals**

390    East Midlands - Derby: 06/MRE04/65.

391    The University of Birmingham Human Biomaterials Resource Centre (National Research  
392    Ethics Service (North West 5): 09/H1010/75.

393    The University Hospitals of North Midlands NHS Trust (National Research Ethics Service  
394    (South Central – Oxford C): 12/SC/0725.

395

396    **Reagents**

397    EZ DNA Methylation Gold kit, Zymo Research, D5005

398    HumanMethylation450 BeadChip arrays, Illumina, WG-314-1003

399    PyroMark Gold Q24 Reagents, Qiagen, 970802

400    SYBR III brilliant green, Agilent, 600882

401

402    **Acknowledgements.**

403    We would like to thank Dr Kim Haworth and Dr Kiren Yacqub-Usman for their support of the  
404    laboratory work.

405    We would like to thank all the West Midlands Consultant Urologists and their units involved  
406    with BCPP, as well as the BCPP research nurses and Margaret Grant, Deborah Bird,  
407    Jennifer Barnwell, Duncan Nekeman and Eline van Roekel.

408

409

## References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *International journal of cancer Journal international du cancer* 2015; 136:E359-86.
2. Kirkali Z, Chan T, Manoharan M, Algaba F, Busch C, Cheng L, Kiemeny L, Kriegmair M, Montironi R, Murphy WM, et al. Bladder cancer: epidemiology, staging and grading, and diagnosis. *Urology* 2005; 66:4-34.
3. Sylvester RJ, van der Meijden AP, Oosterlinck W, Witjes JA, Bouffoux C, Denis L, Newling DW, Kurth K. Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials. *European urology* 2006; 49:466-5; discussion 75-7.
4. Boustead GB, Fowler S, Swamy R, Kocklebergh R, Hounsborne L, Section of Oncology B. Stage, grade and pathological characteristics of bladder cancer in the UK: British Association of Urological Surgeons (BAUS) urological tumour registry. *BJU international* 2014; 113:924-30.
5. Cambier S, Sylvester RJ, Collette L, Gontero P, Brausi MA, van Andel G, Kirkels WJ, Silva FC, Oosterlinck W, Prescott S, et al. EORTC Nomograms and Risk Groups for Predicting Recurrence, Progression, and Disease-specific and Overall Survival in Non-Muscle-invasive Stage Ta-T1 Urothelial Bladder Cancer Patients Treated with 1-3 Years of Maintenance Bacillus Calmette-Guerin. *European urology* 2015.
6. Vedder MM, Marquez M, de Bekker-Grob EW, Calle ML, Dyrskjot L, Kogevinas M, Segersten U, Malmstrom PU, Algaba F, Beukers W, et al. Risk prediction scores for recurrence and progression of non-muscle invasive bladder cancer: an international validation in primary tumours. *PloS one* 2014; 9:e96849.
7. Knowles MA. Molecular pathogenesis of bladder cancer. *International journal of clinical oncology* 2008; 13:287-97.
8. Dudzic E, Goepel JR, Catto JW. Global epigenetic profiling in bladder cancer. *Epigenomics* 2011; 3:35-45.
9. Van Batavia J, Yamany T, Molotkov A, Dan H, Mansukhani M, Batourina E, Schneider K, Oyon D, Dunlop M, Wu XR, et al. Bladder cancers arise from distinct urothelial sub-populations. *Nature cell biology* 2014; 16:982-91.
10. Bakkar AA, Wallerand H, Radvanyi F, Lahaye JB, Pissard S, Lecerf L, Kouyoumdjian JC, Abbou CC, Paire JC, Jaurand MC, et al. FGFR3 and TP53 gene mutations define two distinct pathways in urothelial cell carcinoma of the bladder. *Cancer research* 2003; 63:8108-12.
11. Van Batavia J, Yamany T, Molotkov A, Dan H, Mansukhani M, Batourina E, Schneider K, Oyon D, Dunlop M, Wu XR, et al. Bladder cancers arise from distinct urothelial sub-populations. *Nature cell biology* 2014; 16:982-91, 1-5.
12. Bryan RT, Ward DG. Words of wisdom. Bladder cancers arise from distinct urothelial sub-populations. *European urology* 2015; 67:590-1.
13. McConkey DJ, Lee S, Choi W, Tran M, Majewski T, Lee S, Siefker-Radtke A, Dinney C, Czerniak B. Molecular genetics of bladder cancer: Emerging mechanisms of tumor initiation and progression. *Urologic oncology* 2010; 28:429-40.
14. Castillo-Martin M, Domingo-Domenech J, Karni-Schmidt O, Matos T, Cordon-Cardo C. Molecular pathways of urothelial development and bladder tumorigenesis. *Urologic oncology* 2010; 28:401-8.
15. Knowles MA, Hurst CD. Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity. *Nature reviews Cancer* 2015; 15:25-41.
16. Majewski T, Lee S, Jeong J, Yoon DS, Kram A, Kim MS, Tuziak T, Bondaruk J, Lee S, Park WS, et al. Understanding the development of human bladder cancer by using a whole-organ genomic mapping strategy. *Laboratory investigation; a journal of technical methods and pathology* 2008; 88:694-721.

- 460 17. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis* 2010; 31:27-36.
- 461 18. Baylin SB, Jones PA. A decade of exploring the cancer epigenome - biological and  
462 translational implications. *Nature reviews Cancer* 2011; 11:726-34.
- 463 19. Dawson MA, Kouzarides T. Cancer epigenetics: from mechanism to therapy. *Cell* 2012;  
464 150:12-27.
- 465 20. Kanwal R, Gupta S. Epigenetic modifications in cancer. *Clinical genetics* 2012; 81:303-11.
- 466 21. Wolff EM, Chihara Y, Pan F, Weisenberger DJ, Siegmund KD, Sugano K, Kawashima K, Laird  
467 PW, Jones PA, Liang G. Unique DNA methylation patterns distinguish noninvasive and invasive  
468 urothelial cancers and establish an epigenetic field defect in premalignant tissue. *Cancer research*  
469 2010; 70:8169-78.
- 470 22. Catto JW, Azzouzi AR, Rehman I, Feeley KM, Cross SS, Amira N, Fromont G, Sibony M,  
471 Cussenot O, Meuth M, et al. Promoter hypermethylation is associated with tumor location, stage,  
472 and subsequent progression in transitional cell carcinoma. *Journal of clinical oncology : official*  
473 *journal of the American Society of Clinical Oncology* 2005; 23:2903-10.
- 474 23. Kitchen MO, Bryan RT, Haworth KE, Emes RD, Luscombe C, Gommersall L, Cheng KK, Zeegers  
475 MP, James ND, Devall AJ, et al. Methylation of HOXA9 and ISL1 Predicts Patient Outcome in High-  
476 Grade Non-Invasive Bladder Cancer. *PloS one* 2015; 10:e0137003.
- 477 24. Alvarez-Mugica M, Cebrian V, Fernandez-Gomez JM, Fresno F, Escaf S, Sanchez-Carbayo M.  
478 Myopodin methylation is associated with clinical outcome in patients with T1G3 bladder cancer. *The*  
479 *Journal of urology* 2010; 184:1507-13.
- 480 25. Glossop JR, Nixon NB, Emes RD, Haworth KE, Packham JC, Dawes PT, Fryer AA, Matthey DL,  
481 Farrell WE. Epigenome-wide profiling identifies significant differences in DNA methylation between  
482 matched-pairs of T- and B-lymphocytes from healthy individuals. *Epigenetics : official journal of the*  
483 *DNA Methylation Society* 2013; 8:1188-97.
- 484 26. de Araujo E, Marchi FA, Rodrigues TC, Vieira HC, Kuasne H, Achatz MI, Moredo LF, de Sa BC,  
485 Duprat JP, Brentani HP, et al. Genome-wide DNA methylation profile of leukocytes from melanoma  
486 patients with and without CDKN2A mutations. *Experimental and molecular pathology* 2014; 97:425-  
487 32.
- 488 27. Stefansson OA, Moran S, Gomez A, Sayols S, Arribas-Jorba C, Sandoval J, Hilmarsdottir H,  
489 Olafsdottir E, Tryggvadottir L, Jonasson JG, et al. A DNA methylation-based definition of biologically  
490 distinct breast cancer subtypes. *Molecular oncology* 2015; 9:555-68.
- 491 28. Sanchez-Vega F, Gotea V, Petrykowska HM, Margolin G, Krivak TC, DeLoia JA, Bell DW,  
492 Elnitski L. Recurrent patterns of DNA methylation in the ZNF154, CASP8, and VHL promoters across a  
493 wide spectrum of human solid epithelial tumors and cancer cell lines. *Epigenetics : official journal of*  
494 *the DNA Methylation Society* 2013; 8:1355-72.
- 495 29. Besaratinia A, Cockburn M, Tommasi S. Alterations of DNA methylome in human bladder  
496 cancer. *Epigenetics : official journal of the DNA Methylation Society* 2013; 8:1013-22.
- 497 30. Bryan RT, Kirby R, Mostafid H. Does the nonurologic scientific community understand  
498 urothelial bladder cancer? *European urology* 2014; 66:601-2.
- 499 31. Kelly TK, De Carvalho DD, Jones PA. Epigenetic modifications as therapeutic targets. *Nature*  
500 *biotechnology* 2010; 28:1069-78.
- 501 32. Issa JP, Kantarjian HM. Targeting DNA methylation. *Clinical cancer research : an official*  
502 *journal of the American Association for Cancer Research* 2009; 15:3938-46.
- 503 33. Dedeurwaerder S, Defrance M, Calonne E, Denis H, Sotiriou C, Fuks F. Evaluation of the  
504 Infinium Methylation 450K technology. *Epigenomics* 2011; 3:771-84.
- 505 34. Roessler J, Ammerpohl O, Gutwein J, Hasemeier B, Anwar SL, Kreipe H, Lehmann U.  
506 Quantitative cross-validation and content analysis of the 450k DNA methylation array from Illumina,  
507 Inc. *BMC research notes* 2012; 5:210.
- 508 35. Reinert T, Modin C, Castano FM, Lamy P, Wojdacz TK, Hansen LL, Wiuf C, Borre M, Dyrskjot  
509 L, Orntoft TF. Comprehensive genome methylation analysis in bladder cancer: identification and  
510 validation of novel methylated genes and application of these as urinary tumor markers. *Clinical*

511 cancer research : an official journal of the American Association for Cancer Research 2011; 17:5582-  
512 92.

513 36. Bediaga NG, Acha-Sagredo A, Guerra I, Viguri A, Albaina C, Ruiz Diaz I, Rezola R, Alberdi MJ,  
514 Dopazo J, Montaner D, et al. DNA methylation epigenotypes in breast cancer molecular subtypes.  
515 Breast cancer research : BCR 2010; 12:R77.

516 37. Duong CV, Emes RD, Wessely F, Yacqub-Usman K, Clayton RN, Farrell WE. Quantitative,  
517 genome-wide analysis of the DNA methylome in sporadic pituitary adenomas. Endocrine-related  
518 cancer 2012; 19:805-16.

519 38. Kim YJ, Yoon HY, Kim JS, Kang HW, Min BD, Kim SK, Ha YS, Kim IY, Ryu KH, Lee SC, et al.  
520 HOXA9, ISL1 and ALDH1A3 methylation patterns as prognostic markers for nonmuscle invasive  
521 bladder cancer: array-based DNA methylation and expression profiling. International journal of  
522 cancer Journal international du cancer 2013; 133:1135-42.

523 39. Schubeler D. Function and information content of DNA methylation. Nature 2015; 517:321-  
524 6.

525 40. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, Le JM, Delano D, Zhang L, Schroth GP,  
526 Gunderson KL, et al. High density DNA methylation array with single CpG site resolution. Genomics  
527 2011; 98:288-95.

528 41. Barrera V, Peinado MA. Evaluation of single CpG sites as proxies of CpG island methylation  
529 states at the genome scale. Nucleic acids research 2012; 40:11490-8.

530 42. Naumov VA, Generozov EV, Zaharjevskaya NB, Matushkina DS, Larin AK, Chernyshov SV,  
531 Alekseev MV, Shelygin YA, Govorun VM. Genome-scale analysis of DNA methylation in colorectal  
532 cancer using Infinium HumanMethylation450 BeadChips. Epigenetics : official journal of the DNA  
533 Methylation Society 2013; 8:921-34.

534 43. Karlsson A, Jonsson M, Lauss M, Brunnstrom H, Jonsson P, Borg A, Jonsson G, Ringner M,  
535 Planck M, Staaf J. Genome-wide DNA methylation analysis of lung carcinoma reveals one  
536 neuroendocrine and four adenocarcinoma epitypes associated with patient outcome. Clinical cancer  
537 research : an official journal of the American Association for Cancer Research 2014; 20:6127-40.

538 44. Nagase H, Ghosh S. Epigenetics: differential DNA methylation in mammalian somatic tissues.  
539 The FEBS journal 2008; 275:1617-23.

540 45. Cavallaro U, Christofori G. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer.  
541 Nature reviews Cancer 2004; 4:118-32.

542 46. Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007; 128:683-92.

543 47. Ibragimova I, Dulaimi E, Slifker MJ, Chen DY, Uzzo RG, Cairns P. A global profile of gene  
544 promoter methylation in treatment-naïve urothelial cancer. Epigenetics : official journal of the DNA  
545 Methylation Society 2014; 9:760-73.

546 48. Li Y, Li S, Chen J, Shao T, Jiang C, Wang Y, Chen H, Xu J, Li X. Comparative epigenetic analyses  
547 reveal distinct patterns of oncogenic pathways activation in breast cancer subtypes. Human  
548 molecular genetics 2014; 23:5378-93.

549 49. Gyparakis MT, Basdra EK, Papavassiliou AG. DNA methylation biomarkers as diagnostic and  
550 prognostic tools in colorectal cancer. Journal of molecular medicine 2013; 91:1249-56.

551 50. Guo M, Ren J, House MG, Qi Y, Brock MV, Herman JG. Accumulation of promoter  
552 methylation suggests epigenetic progression in squamous cell carcinoma of the esophagus. Clinical  
553 cancer research : an official journal of the American Association for Cancer Research 2006; 12:4515-  
554 22.

555 51. Kaneda A, Matsusaka K, Sakai E, Funata S. DNA methylation accumulation and its  
556 predetermination of future cancer phenotypes. Journal of biochemistry 2014; 156:63-72.

557 52. Yagi K, Akagi K, Hayashi H, Nagae G, Tsuji S, Isagawa T, Midorikawa Y, Nishimura Y, Sakamoto  
558 H, Seto Y, et al. Three DNA methylation epigenotypes in human colorectal cancer. Clinical cancer  
559 research : an official journal of the American Association for Cancer Research 2010; 16:21-33.

53. Fackler MJ, Umbricht CB, Williams D, Argani P, Cruz LA, Merino VF, Teo WW, Zhang Z, Huang P, Visvanathan K, et al. Genome-wide methylation analysis identifies genes specific to breast cancer hormone receptor status and risk of recurrence. *Cancer research* 2011; 71:6195-207.
54. Sandoval J, Mendez-Gonzalez J, Nadal E, Chen G, Carmona FJ, Sayols S, Moran S, Heyn H, Vizoso M, Gomez A, et al. A prognostic DNA methylation signature for stage I non-small-cell lung cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2013; 31:4140-7.
55. Bennett KL, Karpenko M, Lin MT, Claus R, Arab K, Dyckhoff G, Plinkert P, Herpel E, Smiraglia D, Plass C. Frequently methylated tumor suppressor genes in head and neck squamous cell carcinoma. *Cancer research* 2008; 68:4494-9.
56. Snellenberg S, Cillessen SA, Van Criekinge W, Bosch L, Meijer CJ, Snijders PJ, Steenbergen RD. Methylation-mediated repression of PRDM14 contributes to apoptosis evasion in HPV-positive cancers. *Carcinogenesis* 2014; 35:2611-8.
57. Morris MR, Ricketts CJ, Gentle D, McDonald F, Carli N, Khalili H, Brown M, Kishida T, Yao M, Banks RE, et al. Genome-wide methylation analysis identifies epigenetically inactivated candidate tumour suppressor genes in renal cell carcinoma. *Oncogene* 2011; 30:1390-401.
58. Kalari S, Pfeifer GP. Identification of driver and passenger DNA methylation in cancer by epigenomic analysis. *Advances in genetics* 2010; 70:277-308.
59. De Carvalho DD, Sharma S, You JS, Su SF, Taberlay PC, Kelly TK, Yang X, Liang G, Jones PA. DNA methylation screening identifies driver epigenetic events of cancer cell survival. *Cancer cell* 2012; 21:655-67.
60. Schweikert EM, Devarajan A, Witte I, Wilgenbus P, Amort J, Forstermann U, Shabazian A, Grijalva V, Shih DM, Farias-Eisner R, et al. PON3 is upregulated in cancer tissues and protects against mitochondrial superoxide-mediated cell death. *Cell death and differentiation* 2012; 19:1549-60.
61. Sultan AS, Xie J, LeBaron MJ, Ealley EL, Nevalainen MT, Rui H. Stat5 promotes homotypic adhesion and inhibits invasive characteristics of human breast cancer cells. *Oncogene* 2005; 24:746-60.
62. Zeegers MP, Bryan RT, Langford C, Billingham L, Murray P, Deshmukh NS, Hussain S, James N, Wallace DM, Cheng KK. The West Midlands Bladder Cancer Prognosis Programme: rationale and design. *BJU international* 2010; 105:784-8.
63. Babjuk M, Burger M, Zigeuner R, Shariat SF, van Rhijn BW, Comperat E, Sylvester RJ, Kaasinen E, Bohle A, Palou Redorta J, et al. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder: update 2013. *European urology* 2013; 64:639-53.
64. Blin N, Stafford DW. A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic acids research* 1976; 3:2303-8.
65. Lauss M, Aine M, Sjodahl G, Veerla S, Patschan O, Gudjonsson S, Chebil G, Lovgren K, Ferno M, Mansson W, et al. DNA methylation analyses of urothelial carcinoma reveal distinct epigenetic subtypes and an association between gene copy number and methylation status. *Epigenetics : official journal of the DNA Methylation Society* 2012; 7:858-67.
66. Fryer AA, Emes RD, Ismail KM, Haworth KE, Mein C, Carroll WD, Farrell WE. Quantitative, high-resolution epigenetic profiling of CpG loci identifies associations with cord blood plasma homocysteine and birth weight in humans. *Epigenetics : official journal of the DNA Methylation Society* 2011; 6:86-94.
67. Wessely F, Emes RD. Identification of DNA methylation biomarkers from Infinium arrays. *Frontiers in genetics* 2012; 3:161.
68. Glossop JR, Emes RD, Nixon NB, Haworth KE, Packham JC, Dawes PT, Fryer AA, Matthey DL, Farrell WE. Genome-wide DNA methylation profiling in rheumatoid arthritis identifies disease-associated methylation changes that are distinct to individual T- and B-lymphocyte populations. *Epigenetics : official journal of the DNA Methylation Society* 2014; 9:1228-37.



69. Sen A, Heredia N, Senut MC, Hess M, Land S, Qu W, Hollacher K, Dereski MO, Ruden DM. Early life lead exposure causes gender-specific changes in the DNA methylation profile of DNA extracted from dried blood spots. *Epigenomics* 2015; 7:379-93.
70. Sturn A, Quackenbush J, Trajanoski Z. Genesis: cluster analysis of microarray data. *Bioinformatics* 2002; 18:207-8.
71. Khatri P, Draghici S. Ontological analysis of gene expression data: current tools, limitations, and open problems. *Bioinformatics* 2005; 21:3587-95.
72. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical biochemistry* 1987; 162:156-9.
73. Dudley KJ, Revill K, Whitby P, Clayton RN, Farrell WE. Genome-wide analysis in a murine Dnmt1 knockdown model identifies epigenetically silenced genes in primary human pituitary tumors. *Molecular cancer research : MCR* 2008; 6:1567-74.
74. Al-Azzawi H, Yacqub-Usman K, Richardson A, Hofland LJ, Clayton RN, Farrell WE. Reversal of endogenous dopamine receptor silencing in pituitary cells augments receptor-mediated apoptosis. *Endocrinology* 2011; 152:364-73.
75. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25:402-8.
76. Kitchen MO, Yacqub-Usman K, Emes RD, Richardson A, Clayton RN, Farrell WE. Epidrug mediated re-expression of miRNA targeting the HMGA transcripts in pituitary cells. *Pituitary* 2015.
77. Raponi M, Zhang Y, Yu J, Chen G, Lee G, Taylor JM, Macdonald J, Thomas D, Moskaluk C, Wang Y, et al. Gene expression signatures for predicting prognosis of squamous cell and adenocarcinomas of the lung. *Cancer research* 2006; 66:7466-72.
78. Gardiner-Garden M, Frommer M. CpG islands in vertebrate genomes. *Journal of molecular biology* 1987; 196:261-82.

## Figure Legends

**Figure 1. Array filtering steps.** Summary of the steps implemented for the identification of CpGs hypermethylated in HG-NMIBC. The initial filtering steps (\*) included exclusion of non-significant probe data, probes with missing data and probes located on allsomes. RefSeq (National Center for Biotechnology Information Reference Sequence Database). CpG island based upon the UCSC genome browser definition from Gardiner-Garden and Frommer<sup>78</sup>.

**Figure 2. Unsupervised hierarchical clustering analysis of the 1,057 gene promoter-associated hypermethylated CpGs in HG-NMIBC.** Heatmap and dendrogram of differentially methylated gene promoter-associated CpG sites identified by array analysis. The dendrogram above the heatmap separates normal bladder (green bar,  $n=3$ ) and high-grade-NMIBC bladder tumours (red bar,  $n=21$ ). Each row represents an individual CpG locus, and each column represents a normal control or tumour sample (listed beneath the heatmap). The colour scale beneath the heatmap represents methylation status: unmethylated is yellow ( $\beta$ -value=0.0), and fully methylated is blue ( $\beta$ -value=1.0).

**Figure 3. Heatmap for 25 hypermethylated gene promoter-associated CpG islands.** Pyrosequencing validation of 25 gene promoter-associated CpG islands, identified as frequently differentially methylated in high-grade tumours by 450k BeadChip-array analysis. As indicated above the heatmap, the four normal bladder controls are presented to the left-side of the heatmap, followed by 18 low-intermediate-grade tumours, and 51 high-grade tumours (the combined discovery and investigation cohorts). Each row represents the promoter-associated CpG island of the indicated gene, and each colour block the *mean level of methylation* across the island. The colour scale beneath the heatmap represents methylation status: unmethylated is green (0.0% methylation), and fully methylated is red (100.0% methylated).

**Figure 4. Mean levels of methylation in high-grade tumours relative to low-intermediate-grade tumours and normal bladder.** Top ten genes showing an increase in mean level of methylation (solid red bar) in high-grade tumours (HG,  $n=51$ ) relative to low-intermediate-grade tumours (LG,  $n=18$ ) and in comparison to normal bladder controls (C,  $n=4$ ). Each individual control or tumour sample is shown as an unfilled blue circle. Significant differences in the mean levels of methylation between the low-intermediate- and high-grade tumours, or between control and low-intermediate-grade tumours, are indicated by \*,  $p<0.05$ , or \*\*,  $p<0.005$  (Student's T-test).

**Figure 5. Association of methylation with gene transcript expression in HG-NMIBC.** Tumour transcript expression in unmethylated (UM, unfilled circles) and methylated (M, filled circles) high-grade tumours, relative to normal bladder control (C, unfilled triangles) for the four genes showing significant Spearman's correlation coefficients between promoter methylation and gene expression (*PON3*, *STAT5a*, *VAX2* and *ARHGEF4*;  $p=0.0006$ ,  $p=0.005$ ,  $p=0.013$  and  $p=0.0007$ , respectively). The double-headed arrow represents the threshold for 3-fold reduced expression relative to the mean of the normal controls (solid blue bar); expression at or below this threshold signifies reduced expression in tumour samples.

## Tables

Gene Symbol	METHYLATION FREQUENCY			MEAN LEVEL OF METHYLATION		
	High-grade	Low-intermediate- grade	<i>P</i> value	High-grade	Low-intermediate- grade	<i>P</i> value
	<i>Number (%)</i>	<i>Number (%)</i>		<i>(%)</i>	<i>(%)</i>	
<i>ATP5G2</i>	37/51 (72.5)	6/18 (33.3)	<b>0.005</b>	51.04	30.20	<b>0.029</b>
<i>VAX2</i>	13/51 (25.5)	0/18 (0.0)	<b>0.015</b>	32.31	19.56	<b>0.004</b>
<i>IRX1</i>	37/51 (72.5)	8/18 (44.4)	<b>0.045</b>	49.47	38.70	0.067
<i>INSRR</i>	29/51 (56.9)	5/18 (27.8)	0.054	24.06	24.06	<b>0.028</b>
<i>IRF8</i>	25/51 (49.0)	4/18 (22.2)	0.057	26.13	17.99	0.157
<i>PRDM14</i>	45/51 (88.2)	12/18 (66.7)	0.066	60.14	46.06	<b>0.029</b>
<i>VSX1</i>	44/51 (86.3)	12/18 (66.7)	0.086	56.37	38.26	<b>0.0004</b>
<i>TFAP2b</i>	22/51 (43.1)	4/18 (22.2)	0.160	32.25	17.68	<b>0.047</b>
<i>PRRX1</i>	27/51 (52.9)	7/18 (38.9)	0.413	47.03	34.36	<b>0.041</b>
<i>HIST1H4F</i>	42/51 (82.4)	13/18 (72.2)	0.496	59.46	41.91	<b>0.017</b>

**Table 1. Genes showing the greatest methylation increase in high-grade relative to low-intermediate-grade NMIBC tumours.** Top ten genes showing an increase in frequency of methylation (left side of table), and/or an increase in mean level of methylation (right side of table) in high-grade tumours relative to low-intermediate-grade tumours. For the left side of the table, the number and proportion of tumours methylated are displayed for the low-intermediate- and high-grade cohorts, with *p*-value (Fishers exact, *p*<0.05 significant). For the right side of the table, the mean level of methylation across the low-intermediate- and high-grade tumour cohorts are displayed with *p*-value (Student's T-Test, *p*<0.05 significant). Statistically significant *p*-values are displayed in bold.

## Supplemental Data

**Figure S1. Technical validation of 450k BeadChip-array data.** Correlation between array-derived  $\beta$ -values (x-axis) and methylation percentage as determined by Pyrosequencing (y-axis) for 5 CpGs (cg07778029, cg14456683, cg01227537, cg05661282 and cg26465391) across 24 samples is shown. Spearman-rank correlation coefficient  $r=0.912$ ;  $p<0.00001$ .

**Table S1. Sample characteristics.**

**Table S2. List of 256 differentially methylated genes.**

**Table S3. Methylation in discovery and investigation high-grade tumour cohorts.**

**Table S4. Frequency and mean levels of methylation in 25 genes for high- and low-intermediate-grade tumours.**

**Table S5. Gene Ontology and KEGG pathway annotation lists.**

**Table S6. Primer sequences.**